# The effect of inhibition of nitric oxide synthase on aluminium-induced toxicity in the rat brain

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**Abstract.** The goal of the present study was to examine the effectiveness of a non-specific nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME) to modulate the toxicity of aluminium chloride ($AlCl_3$). Rats were killed at 3 h and at 30 days after treatments and the striatum was removed. Nitrite, superoxide, superoxide dismutase activity, malondialdehyde and reduced glutathione were determined. $AlCl_3$ exposure promoted oxidative stress in the striatum. The biochemical changes observed in neuronal tissues show that aluminium acts as pro-oxidant, while the NOS inhibitor exerts antioxidant action in $AlCl_3$-treated rats. We conclude that L-NAME can efficiently protect neuronal tissue from $AlCl_3$-induced toxicity.

**Key words:** Aluminium — L-NAME — Nitric oxide — Oxidative stress — Striatum

## Introduction

Aluminium (Al) is a neurotoxic metal that contributes to the progression of several neurodegenerative diseases (Ferreira et al. 2008). Al influx into brain tissues involves transferrin receptor-mediated endocytosis and a more rapid process which transports low molecular weight Al species. Reports have documented ghost-like neurons with Al deposition within their cytoplasmic and nuclear vacuoles (Platt et al. 2001; Miu et al. 2003). The hippocampus contains extracellular accumulations of Al and amyloid surrounded by nuclei of degenerating cells which are termed neuritic plaques (Drago et al. 2008). Al promotes the formation and accumulation of insoluble β amyloid (Aβ) and hyperphosphorylated tau. In addition, Al mimics the deficit of cortical cholinergic neurotransmission seen in several neurodegenerative diseases (Exley 2007).

Excessive microglial activation contributes to the neurodegenerative process by releasing potent cytotoxic substances including the free radical nitric oxide (NO•) (Hara et al. 2007). Chronic exposure to Al impairs glutamate-induced activation of NO synthase (NOS) and NO•-induced activation of guanylate cyclase (Cucarella et al. 1998). Cortical nitroxidergic neurons and granule cells are specific targets of Al-dependent neurotoxicity (Rodella et al. 2001).

NOS is present in the mammalian brain as three different isoforms, two constitutively-active (neuronal – nNOS, and endothelial – eNOS) and one inducible (iNOS). All three isoforms are aberrantly expressed during Al intoxication resulting in elevated NO levels. Elevated NO levels contribute to neurodegenerative process *via* different mechanisms including oxidative stress and the activation of intracellular signalling mechanisms (Luth et al. 2001).

Under physiological and pathological conditions and in the presence of molecular oxygen, reactive oxygen species (ROS) production such as the superoxide anion radical ($O_2^{•-}$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical (HO•) occurs (Scandalios 2005). Elevated iron concentration in the brain promotes HO• formation through the Fenton reaction, while $O_2^{•-}$ reacts with NO• to form the harmful peroxynitrite anion ($ONOO^-$). All together, the above-mentioned processes contribute to oxidative stress within the brain (Johnson 2001).

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Pro-oxidants attack lipids within cell membranes *via* the process termed lipid peroxidation. The concentration of malondialdehyde (MDA) has been used as an index of lipid peroxidation in situations of Al exposure (Tanino et al. 2000).

Oxygen derivatives are eliminated *via* a coordinated antioxidant defence system comprising enzymes (glutathione (GSH) peroxidase, superoxide dismutase (SOD) and catalase) and water- or fat-soluble non-enzymatic antioxidants (vitamins C and E, GSH and selenium) (Stanczyk et al. 2005).

Changes in neurite morphology and cell death are partly reduced by attenuation of the effects of $NO^\bullet$ (Yang et al. 1998). Our previous results demonstrated positive effects of NOS inhibitors on the development of neurotoxicity (Vasiljević et al. 2002; Stevanović et al. 2008). In view of the above, the present study was undertaken to examine whether one or more oxidative stress status parameters (nitrite, $O_2^{\bullet-}$, SOD activity, MDA and GSH) change after intracerebral injections of aluminium chloride ($AlCl_3$) into rats and if they are modulated by pre-treatment of the rats with N-nitro-L-arginine methyl ester (L-NAME), a non-specific NOS inhibitor.

## Materials and Methods

### Animals

Adult male Wistar rats, weighing $500 \pm 50$ g, were used for the experiments. Two or three animals were housed per cage in an air-conditioned room at a temperature of $23 \pm 2$ºC with $55 \pm 10\%$ humidity and with light intervals of 12 h/day (07.00–19.00 h). The animals were given a commercial rat diet and tap water *ad libitum*.

Animals used for all experimental procedures were handled in strict accordance with the NIH Guide for Care and Use of Laboratory Animals, 1985.

### Reagents

All chemicals were of analytical grade or better. $AlCl_3$, L-NAME, EDTA, epinephrine and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA), thiobarbituric acid reagent (TBAR) and 15% trichloroacetic acid were from Merck (Darmstadt, Germany), carbonate buffer was from Serva (Feinbiochemica, Germany) and saline solution (0.9% sodium chloride) was provided by the hospital pharmacy (Military Medical Academy, Belgrade). All solutions of drugs were prepared on the day of experiment.

### Experimental procedures

The rats were intraperitoneally anesthetised with sodium pentobarbital (45 mg/kg body weight – b.w.) before intra-

hippocampal administration of the following: the control group ($n = 8$) was treated with 10 μl of saline solution; the $AlCl_3$ group ($n = 15$) was treated with $AlCl_3$ in one single dose ($3.7 \times 10^{-4}$ g/kg b.w. in 0.01 ml of deionised water); the L-NAME+$AlCl_3$ group ($n = 10$) was pre-treated with L-NAME in one single dose ($1 \times 10^{-4}$ g dissolved in saline solution) before $AlCl_3$ administration; the L-NAME group ($n = 10$) was treated with L-NAME in one single dose ($1 \times 10^{-4}$ g dissolved in saline solution) before saline solution administration. L-NAME was administered immediately before the $AlCl_3$/saline solution. L-NAME's half life varies from 7.5 min up to 222 min (Mitsube et al. 2002).

Using a stereotaxic instrument for small animals, the drugs were administered *via* a Hamilton microsyringe and injected into the CA1 sector of the hippocampus (coordinates: 2.5 A; 4.2 L; 2.4 V) (König and Klippel 1963). In all the treated animals the injected intracerebral volume was 10 μl and it was always injected into the same left side.

Each of the four experimental groups (defined above according to drug treatment) were divided into two subgroups. At 3 h and 30 days after treatment the rats were decapitated. The heads were immediately frozen in liquid nitrogen and stored at –70ºC until use. A crude mitochondrial fraction from the striatum was used for the biochemical analyses (Gurd et al. 1974).

### Biochemical analyses

After deproteinisation $NO^\bullet$ was quantitated by measuring nitrite and nitrate concentrations. Nitrites were directly assayed spectrophotometrically at 492 nm using the colourimetric method described by Griess (Griess reagent: 1.5% sulphanilamide in 1 mol/l HCl containing 0.15% N-(1-naphthyl)ethylendiamine dihydrochloride). Nitrates had to be converted into nitrites by cadmium reduction (Navarro-Gonzalvez et al. 1998).

Superoxide anion was determined *via* the reduction of nitroblue-tetrazolium (Merck, Darmstadt, Germany) in alkaline buffer (oxygen-free), with kinetic analysis performed at 550 nm (Auclair and Voisin 1985).

SOD activity was measured spectrophotometrically as inhibition of spontaneous epinephrine auto-oxidation at 480 nm. Enzyme activity in samples was monitored in a bicarbonate buffer (50 mmol/l, pH 10.2) containing 0.1 mmol/l EDTA after the addition of 10 mmol/l epinephrine (Sun and Zigman 1978).

The lipid peroxidation index was determined according to the quantity of MDA produced. TBAR (15% trichloroacetic acid + 0.375% TBA + 0.25% mol HCl) reacts with MDA originating from polysaturated fatty acid peroxidation. The product of the reaction, MDA, was measured spectrophotometrically at 533 nm (Villacara et al. 1989).

Reduced GSH was determined using 5,5-dithiobis-2-nitrobenzoic acid (36.9 mg in 10 ml of methanol) which reacts

with aliphatic thiol compounds in Tris-HCl buffer (0.4 mol/l, pH 8.9) generating a yellow coloured p-nitrophenol anion. Colour intensity was spectrophotometrically determined at 412 nm. Brain tissue was prepared in 10% sulphosalicylic acid for GSH determination (Anderson 1986).

The protein content in the rat brain homogenates (striatum, ipsilateral and contralateral) was measured by the method of Lowry using bovine serum albumin as a standard (Lowry et al. 1951).

### Data presentation and analysis

Data are expressed as means ± S.D. Differences were deemed statistically significant if $p < 0.05$ via either the Student's $t$-test or ANOVA, the latter followed by Tukey's test.

### Results

#### Nitrite concentration in the rat striatum

Three hours after $AlCl_3$ injection the nitrite concentration increased bilaterally in the striatum, compared to control-injected rats (Fig. 1A). In the L-NAME+$AlCl_3$ group, the bilateral nitrite concentration was lower in the striatum, compared to the $AlCl_3$-treated group (Fig. 1A). Thirty days after L-NAME+$AlCl_3$ injection, the bilateral nitrite concentration was lower compared to both control- and $AlCl_3$-injected rats (Fig. 1B). Three hours after L-NAME injection, the nitrite concentration increased bilaterally in the striatum, compared to both control- and L-NAME+$AlCl_3$-injected rats (Fig. 1A). However, after 30 days L-NAME injection resulted in lower nitrite concentrations in both the ipsi- and contralateral striatum compared to control- and $AlCl_3$-injected rats.

#### Superoxide production in the rat striatum

$AlCl_3$ injection resulted in higher $O_2^{\bullet-}$ production both after 3 h and after 30 days in both the ipsi- and contralateral striatum, compared to control rats ($p < 0.05$). In the L-NAME+$AlCl_3$ group $O_2^{\bullet-}$ production after 3 h decreased bilaterally in the striatum compared to the $AlCl_3$-treated group (Fig. 2A). Furthermore, after 30 days in the L-NAME+$AlCl_3$ group $O_2^{\bullet-}$ production decreased bilaterally in the same brain structure compared to both the control group as well as to the $AlCl_3$-injected group (Fig. 2B). Three hours after L-NAME injection, $O_2^{\bullet-}$ production decreased bilaterally in the striatum compared to $AlCl_3$-injected rats (Fig. 2A). Thirty days after L-NAME injection, $O_2^{\bullet-}$ production decreased bilaterally in the striatum compared to control-injected rats and compared to $AlCl_3$-injected rats. In contrast, $O_2^{\bullet-}$ production increased bilaterally in this
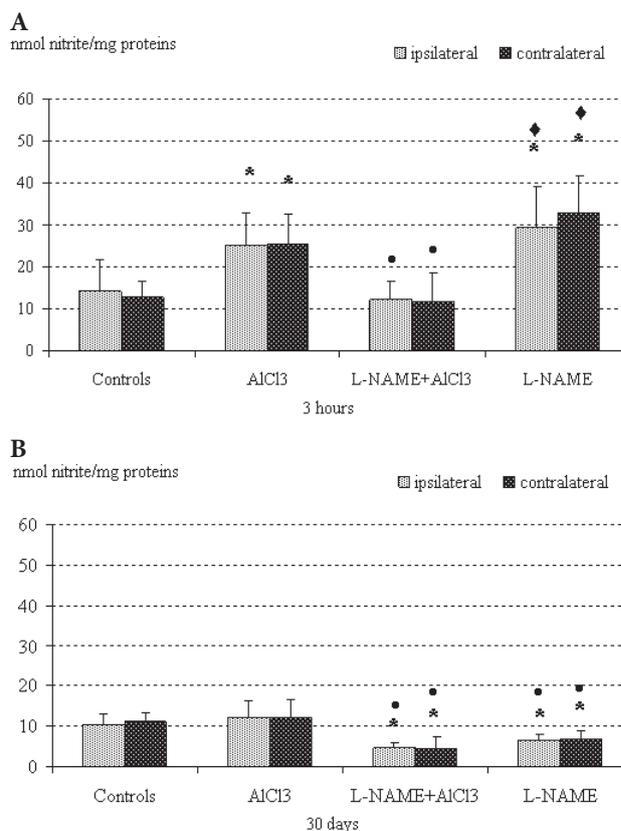


**Figure 1.** The effect of intrahippocampal drug administration on the concentration of nitrite (nmol/l nitrite/mg protein) in the rat ipsilateral and contralateral striatum at different times post injection: 3 h (A) and 30 days (B). Data are means ± S.D. of 10 animals. ⋆ statistically significant difference between treated ($AlCl_3$-, L-NAME+$AlCl_3$- and L-NAME-treated) and control (sham-operated) animals ($p < 0.05$); ● statistically significant difference between treated (L-NAME+$AlCl_3$- and L-NAME-treated) and $AlCl_3$-treated animals ($p < 0.05$); ◆ statistically significant difference between L-NAME-treated and L-NAME+$AlCl_3$-treated animals ($p < 0.05$).

brain structure compared to the L-NAME+$AlCl_3$-treated group (Fig. 2B).

#### SOD activity in the rat striatum

After both 3 h and 30 days, $AlCl_3$ injection resulted in lower SOD activity, compared to the control group. However, the difference was not statistically significant (Fig. 3A,B). After both 3 h and 30 days of L-NAME+$AlCl_3$ injection, lower SOD activity was found, compared to both control and $AlCl_3$-injected rats ($p < 0.05$). After both 3 h and 30 days of L-NAME application, lower SOD activity was found bilaterally in the striatum compared to both control and $AlCl_3$-injected rats. In contrast, higher SOD activity was

**A**

μmol red. NBT/min/mg proteins



**B**

μmol red. NBT/min/mg proteins
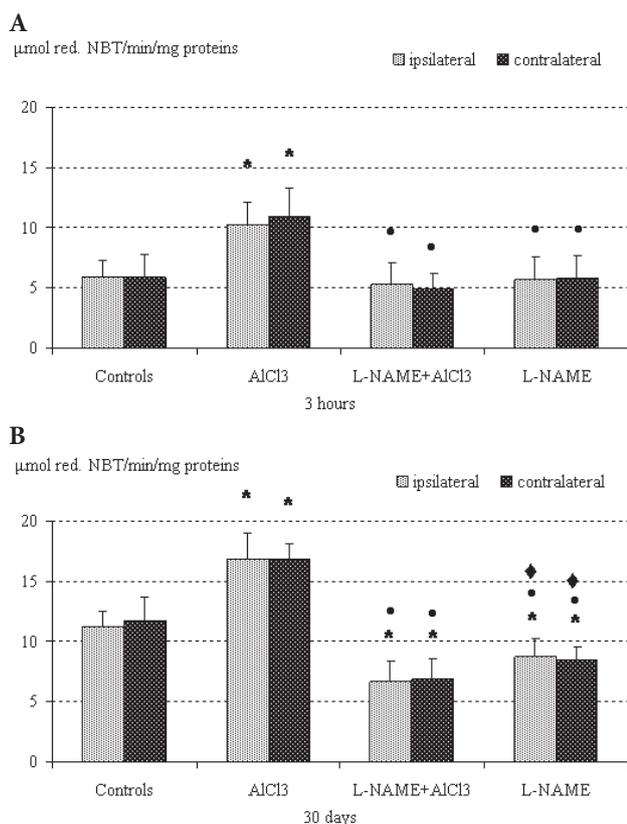


**Figure 2.** The effect of intrahippocampal drug administration on the concentration of $O_2^{\bullet-}$ (μmol/l red. NBT/min/mg proteins) in the rat ipsilateral and contralateral striatum at different survival times post injection: 3 h (A) and 30 days (B). Data are means ± S.D. of 10 animals. $^\star$ statistically significant difference between treated (AlCl$_3$-, L-NAME+AlCl$_3$- and L-NAME-treated) and control (sham-operated) animals ($p < 0.05$); $^\bullet$ statistically significant difference between treated (L-NAME+AlCl$_3$- and L-NAME-treated) and AlCl$_3$-treated animals ($p < 0.05$); $^\blacklozenge$ statistically significant difference between L-NAME-treated and L-NAME+AlCl$_3$-treated animals ($p < 0.05$).

found in both ipsi- and contralateral striatum compared to the L-NAME+AlCl$_3$-injected group of rats (Fig. 3A,B).

*MDA concentration in the rat striatum*

After both 3 h and 30 days post AlCl$_3$ injection, increased MDA concentration bilaterally in the striatum was apparent compared to control rats (Fig. 4A,B). L-NAME+AlCl$_3$ administration resulted in a decrease in the MDA concentration bilaterally in the same brain structure after both 3 h and 30 days compared to AlCl$_3$-injected rats. After 3 h, L-NAME administration resulted in a higher MDA concentration in the ipsilateral striatum compared to L-NAME+AlCl$_3$-injected rats and bilaterally in the same brain structure compared to control-treated rats. After 3 h,

**A**

U/mg proteins



**B**
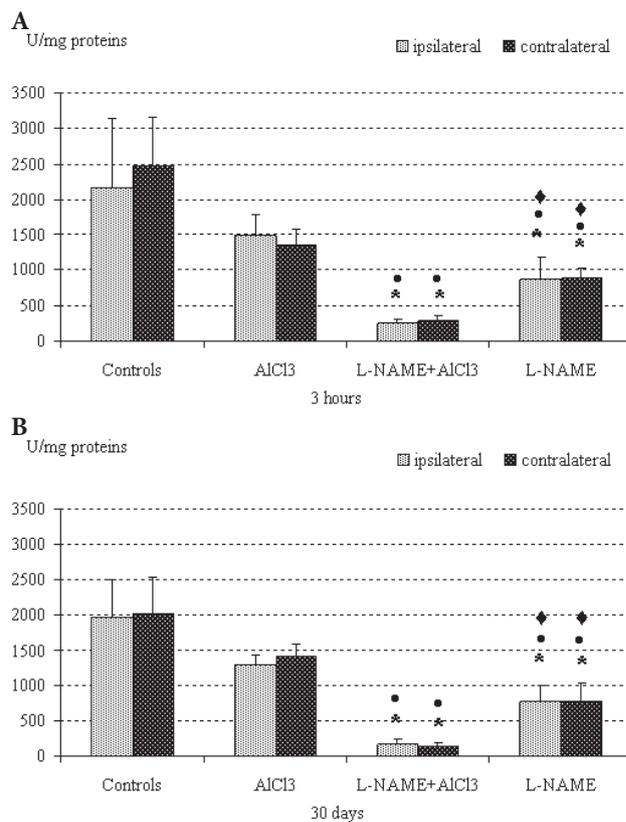
U/mg proteins



**Figure 3.** The effect of intrahippocampal drug administration on SOD activity (U/mg proteins) in the rat ipsilateral and contralateral striatum at different survival times post injection: 3 h (A) and 30 days (B). Data are means ± S.D. of 10 animals. $^\star$ statistically significant difference between treated (L-NAME+AlCl$_3$- and L-NAME-treated) and control (sham-operated) animals ($p < 0.05$); $^\bullet$ statistically significant difference between treated (L-NAME+AlCl$_3$- and L-NAME-treated) and AlCl$_3$-treated animals ($p < 0.05$); $^\blacklozenge$ statistically significant difference between L-NAME-treated and L-NAME+AlCl$_3$-treated animals ($p < 0.05$).

L-NAME resulted in a lower MDA concentration in both the ipsi- and contralateral striatum compared to AlCl$_3$-injected rats (Fig. 4A). After 30 days, L-NAME injection resulted in a lower MDA concentration compared to both control and AlCl$_3$-injected rats (Fig. 4B).

*Reduced GSH content in the rat striatum*

The GSH concentration was higher (bilaterally in the striatum) 3 h post AlCl$_3$ injection compared to control-injected rats (Fig. 5A). After 3 h, L-NAME+AlCl$_3$ administration resulted in a lower GSH concentration in both the ipsi- and contralateral striatum compared to the AlCl$_3$-treated group. In addition, 30 days after L-NAME+AlCl$_3$ administration, the GSH concentration decreased bilaterally in the striatum

**A**

nmol MDA/h/mg proteins



**B**

nmol MDA/h/mg proteins
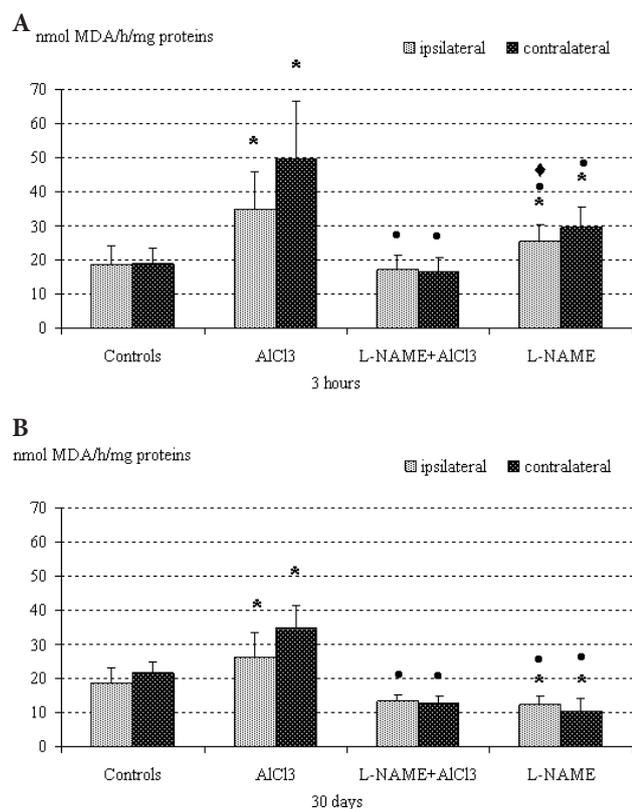


**Figure 4.** The effect of intrahippocampal drug administration on lipid peroxidation (nmol/l MDA/h/mg proteins) in the rat ipsilateral and contralateral striatum at different survival times post injection: 3 h (A) and 30 days (B). Data are means ± S.D. of 10 animals. ✶ statistically significant difference between treated (AlCl₃- and L-NAME-treated) and control (sham-operated) animals (*p* < 0.05); • statistically significant difference between treated (L-NAME+AlCl₃- and L-NAME-treated) and AlCl₃-treated animals (*p* < 0.05). ✦ statistically significant difference between L-NAME-treated and L-NAME+AlCl₃-treated animals (*p* < 0.05).

**A**

nmol GSH/mg proteins



**B**

nmol GSH/mg proteins



**Figure 5.** The effect of intrahippocampal drug administration on reduced GSH concentration (nM GSH/mg proteins) in the rat ipsilateral and contralateral striatum at different survival times post injection: 3 h (A) and 30 days (B). Data are means ± S.D. of 10 animals. ✶ statistically significant difference between treated (AlCl₃-, L-NAME+AlCl₃- and L-NAME-treated) and control (sham-operated) animals (*p* < 0.05); • statistically significant difference between treated (L-NAME+AlCl₃- and L-NAME-treated) and AlCl₃-treated animals (*p* < 0.05); ✦ statistically significant difference between L-NAME-treated and L-NAME+AlCl₃-treated animals (*p* < 0.05).

compared to both control and AlCl₃-injected rats (Fig. 5B). After 3 h, L-NAME administration resulted in a lower GSH concentration bilaterally in the striatum compared to control, AlCl₃- and L-NAME+AlCl₃-injected rats. However, after 30 days, L-NAME injection resulted in a higher concentration of GSH bilaterally in the striatum compared to control, AlCl₃- and L-NAME+AlCl₃-injected rats (Fig. 5B).

**Discussion**

Extensive afferents from all areas of the cortex and the thalamus represent the most important source of excitatory amino acids, whereas the nigrostriatal pathway and intrinsic circuits provide the striatum with dopamine,

acetylcholine, GABA, NO and adenosine. All these neurotransmitters interact in a voltage-dependent manner to regulate the efficacy of synaptic transmission within this circuit (Calabresi et al. 2000).

Our findings that AlCl₃ injection resulted in increased nitrite after 3 h bilaterally in the striatum but without change after 30 days bilaterally in this brain structure compared to control rats is in accordance with previously published data (Tohgi et al. 1998) demonstrating increased NO•concentration and oxidation in early stages of disease, while it was decreasing with elevating loss of neurons.

Increased O₂•⁻ production bilaterally in the striatum 3 h after AlCl₃ injection is in agreement with a published article regarding Al-mediated oxidative stress. Pro-oxidant properties of Aβ have been found in senile plaques and discussed by

Yang et al. (1999). In addition, free radical production in the brain could be stimulated by the presence of low molecular weight iron-containing compounds which are known Fenton reaction promoters.

The product of the reaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ ($ONOO^-$) is a strong oxidising and nitrating agent which can react with all classes of biomolecules (Domico et al. 2007). In the central nervous system (CNS) it can be generated by microglial cells activated by pro-inflammatory cytokines or Aβ and by neurons when $ONOO^-$ directly contributes to the initiation of the neurodegenerative process (Torreilles et al. 1999; Wakselman et al. 2008).

Intracerebral administration of $AlCl_3$ resulted in increased $O_2^{\bullet-}$ production after 30 days in the ipsi- and contralateral striatum. A report has indicated that microglia are the CNS macrophages and are the primary cellular component of plaques that contribute to the oxidative stress associated with chronic neurodegeneration (Colton et al. 2000). The effect of Aβ peptides on $O_2^{\bullet-}$ production was not associated with a concomitant increase in $NO^{\bullet}$ concentration. Although statistically insignificant, $AlCl_3$ injection caused a decrease in SOD activity. The latter is in accordance with increased $O_2^{\bullet-}$ production in the same brain structure, indicating conditions favouring oxidative stress.

After both 3 h and 30 days post intrahippocampal $AlCl_3$ injection, the concentration of MDA increased bilaterally in the striatum. Previous data have demonstrated that Al affects $Ca^{2+}$ flux and general homeostasis, increases ROS and facilitates membrane lipid peroxidation, which together can contribute to neurotoxicity induced by other neurotoxicants (Mundy et al. 1997).

Furthermore, we demonstrated that after 3 h $AlCl_3$ injection increased the concentration of GSH bilaterally in the striatum. All the detrimental effects of $ONOO^-$ can be successfully attenuated by the thiol-containing tripeptide antioxidant GSH (Burdo et al. 2008). Peroxynitrite is a powerful oxidant as it is capable of chemically modifying both membrane and cytosolic proteins which affects both their physical and chemical nature properties (Koppal et al. 1999). Not surprisingly, we found increased GSH concentration 3 h post $AlCl_3$ injection. Other parameters confirmed that oxidative stress processes were operative at this time point.

Our biochemical observations are of particular interest especially when taking in consideration the fact that interactions between basalocortical and intracortical NOS neurons are involved in the spatial and temporal regulation of cortical perfusion following basal forebrain activation (Tong and Hamel 2000). In addition, Al accumulation in the brain can modify neuronal signal transduction pathways associated with glutamate-induced activation of NOS and $NO^{\bullet}$-induced activation of the cGMP generating enzyme, guanylate cyclase. These findings suggest that glutathione-$NO^{\bullet}$-cGMP pathway impairment in the brain may be responsible for some Al-induced neurological alterations (Canales et al. 2001).

The decreased bilateral nitrite concentration in the striatum at 30 days post L-NAME+$AlCl_3$-injected rats (compared to $AlCl_3$-injected rats) suggests that L-NAME suppresses nitrite production and decreases neuron impairment through N-methyl-D-aspartic acid receptors.

Decreased $O_2^{\bullet-}$ production, decreased SOD activity and decreased MDA concentration bilaterally in the striatum at both 3 h and 30 days after L-NAME+$AlCl_3$ application (compared to $AlCl_3$-treated animals) confirm antioxidative effects if the NOS inhibitor. In the same experimental group of animals, we also noted decreased GSH content. Therefore, our results suggest the importance of GSH in the glutathionylation process as a crucial antioxidative defence mechanism against irreversible protein impairment (Giustarini et al. 2004).

The observed bilateral increase in NO concentration in the striatum, compared to the control group of rats, 3 h after L-NAME application was unexpected because of its inhibitory potential. However, in some circumstances L-NAME may contribute to $NO^{\bullet}$ donation, serving as an arginine analog (Chakraborti et al. 2008). Furthermore, the inhibition of inducible NOS expression by L-NAME prevented an increase in nitrogen intermediates and GABA release, but not in glutamate release (Waldmeier et al. 2008). In contrast, our results concerning reduced concentrations of both NO concentration and $O_2^{\bullet-}$ 30 days after L-NAME application, compared to $AlCl_3$-treated rats, suggest long-term inhibition of $NO^{\bullet}$ synthesis by L-NAME, which therefore fortifies its antioxidative potential.

Increased MDA concentration in tune with both decreased SOD activity and GSH concentration bilaterally in the striatum 3 h post L-NAME application, compared to control rats, all together contribute to oxidative development at such an early time point. Thirty days post L-NAME application resulted in an improvement in oxidative stress status development parameters (decreased MDA and increased GSH concentration). L-NAME could be a potential arginine analog, therefore it could have some toxic properties similar to those of $AlCl_3$. MDA may contribute to the protective effect of L-NAME in $AlCl_3$-treated rats, in contrast to $AlCl_3$'s and L-NAME's effects by themselves.

In conclusion, our results indicated that $NO^{\bullet}$-mediated neurotoxicity spread temporally and spatially in the striatum after intrahippocampal $AlCl_3$ administration and that L-NAME has potential neuroprotective roles.

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